REACTION OF ALLICIN-RELATED COMPOUNDS WITH PROTEIN¹

ISAMU UTSUMI, KIYOSHI HARADA AND KEIICHI KOHNO²

Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd., Osaka

(Received April 1, 1964)

Fujiwara *et al.* (1-2) reported that allacin was bound with egg albumin or blood protein *in vitro* and also with body constituents *in vivo*. They assumed that allacin was not chemically bound with the SH groups of protein, but it was additionally bound forming a complex. Allacin was not liberated from the "complex" by washing with water or saturated ammonium sulfate solution, but could be easily separated by extracting with ethanol in which allacin is easily soluble. When protein is deprotenized by metaphosphoric acid or salted out by ammonium sulfate from the mixed system of a relatively hydrophobic substance as allacin and dissolved protein, there is a possibility of enfolding of allacin. Without fractional determination and identification of the unreacted allacin, the allacin which could not be separated by washing alone, cannot be ascribed to the formation of complex with the protein. In the previous paper (3) it was confirmed that symmetric disulfides, such as thiamine disulfide (TDS) or *O*-benzoylthiamine disulfide (BTDS), reacted with the SH groups of protein, forming a protein-S-S-thiamine type compound. Allacin homologs are known to react easily with L-cysteine, forming *S*-alkyl-mercapto-L-cysteine (4-5). It is therefore expected that the SH groups of egg or serum albumin can also easily react with allacin homologs since they have a specific reactivity for the SH group of cysteine. In order to solve these questions the interaction of allacin homologs, $C_3H_7SSC_3H_7$ (I) or $C_3H_7SSOC_3H_7$ (II), with egg albumin was investigated. It was recognized that each of I and II reacted with the SH group of egg albumin, forming a chemically bound compound, protein-S-S- C_3H_7 , though the reaction mechanism were somewhat different between the two compounds.

EXPERIMENTAL

1. Materials

$C_3H_7SSC_3H_7$ (I) and $C_3H_7SSOC_3H_7$ (II)—I was redistilled and the fraction having a boiling-point of 97-98° (30 mg Hg) was used. II was prepared by the procedure of Yoshimura (6) as follows. To a solution of 600 mg I in 4 ml glacial acetic acid was added 0.4 ml of 30 % hydrogen peroxide and the mixture was allowed to stand at room temperature for 24 hours. It was diluted

¹ Studies on Interaction of Thiamine or its Related Compounds with Protein. VII

Preliminarily reported at the 138th Meeting of Vitamin B Research Committee (8).

² 内海 勇, 原田 清, 河野啓一.

with ethanol up to 50 ml, and was employed as a 0.08 *M* stock solution. In the reaction with protein, the stock solution was neutralized under ice cooling with *N* NaOH and a 0.04 *M* solution was used in the experiment.

Egg Albumin—Egg albumin powder, Difco, was dissolved to make a 5 % solution. A small amount of insoluble material was removed by centrifugation at 4000 rpm for 15 minutes.

2. Reaction and Deproteinization

To 5 ml of 5 % egg albumin (in case of urea denaturation 6 g urea was added) were added 4 ml of 0.5 *M* phosphate buffer (pH 7.4), 0.8 ml of 0.1 *M* I solution in ethanol or 2 ml of 0.04 *M* II solution in 50 % ethanol, and water was added up to 15 ml. The mixture was incubated at 37° for one hour with agitation. In order to remove the unreacted I or II, deproteinization was carried out as follows. To the reaction mixture 30 ml of ethanol and 5 ml of 10 % metaphosphoric acid were added. The protein was precipitated by centrifugation at 4000 rpm for 10 minutes, the precipitate was suspended in 40 ml of ethanol and the whole was heated at 60° for 15 minutes, followed by centrifugation. This extraction procedure was repeated three times using 40 ml of ethanol. The protein fraction thus obtained was diluted to a suitable volume with water for further experiments.

3. Assay of Protein-bound C_3H_7SH and Unreacted I and II

To each sample, containing less than 50 μ g as C_3H_7SH , 5 ml of 0.5 *M* phosphate buffer, pH 8.0, and 20 mg of cysteine hydrochloride were added and the final volume was made up to 12 ml with water. After incubation at 37° for one hour, the mixture was acidified with hydrochloric acid and the liberated C_3H_7SH was extracted with 10 ml of chloroform. 2 to 5 ml of the extract was placed into a glass-stoppered tube and chloroform was added up to about 10 ml. Then 10 ml of 3×10^{-5} *M* $HgCl_2$ in *N* H_2SO_4 solution was added and the whole was vigorously agitated for 2 minutes. 5 ml of the water layer was taken and the remaining $HgCl_2$ was determined by the dithizon method (7), and the $HgCl_2$ consumed was calculated. The amount of C_3H_7SH was determined according to the following formulae.

1 ml of 3×10^{-5} *M* $HgCl_2$ corresponds to 2.28 μ g C_3H_7SH

One mole of C_3H_7SH was found to consume 0.95 ± 0.05 mole of $HgCl_2$ in the range of 2 to 20 μ g of the mercaptan under the condition given above, and a small amount of alkylmercaptan could be determined with 95 ± 5 % recovery. But the recoveries were about 60%, when I or II was determined after reduction with cysteine. Several reducing methods were tried but they were all unsatisfactory. When C_3H_7SH was treated with cysteine, the recovery was also lowered than that without cysteine treatment. However, this assay method is more sensitive than any other methods and the procedure is simple. Therefore, it was adopted for the following experiments. The remained II in the supernatant was also determined from the amount of the masked thiamine according to Fujiwara *et al.* (2)

RESULTS AND DISCUSSION

1. Separation of Remaining I or II in the Protein Fraction and Determination of Bound Propylmercaptan

After incubation of native or urea-denatured egg albumin with I or II, ethanol or metaphosphoric acid was added and the protein fraction collected by centrifugation was extracted with hot ethanol several times and the amount of I or II contained in each extract was assayed after reduction to C_3H_7SH . The amount of II was also determined from the amount of masked thiamine. The results are illustrated in Figs. 1 and 2. Deproteinization and extraction with ethanol in

FIG. 1 Ethanol Extraction of the Reaction Product of Egg Albumin with I
●, denatured protein with I; ○, native protein with I

FIG. 2 Ethanol Extraction of the Reaction Product of Egg Albumin with II
●, denatured protein with II; ○, denatured protein with II (determined from the amount of masked thiamine); ×, native protein with II (determined from the amount of masked thiamine)

which these disulfides are readily soluble, followed by deproteinization with metaphosphoric acid and further extraction with hot ethanol 3 to 4 times is assumed to be capable of completely eliminating the occurrence of "enfolding" of the unchanged I or II. Each protein was then treated with cysteine and amount of C_3H_7SH which was expected to have been formed, was determined and a significant amount of the mercaptan was detected. (Figs. 1 and 2). However, in the case of the reaction of I with the native protein, the amount of C_3H_7SH thus liberated was very small. In the supernatant, a large amount of C_3H_7SH was detected after incubation of the denatured protein with I, but scarcely any mercaptan was found after incubation with II. From these results it was assumed that I reacted with the SH- groups of the denatured protein, whereas II reacted

with the SH- groups of the native or denatured protein, producing the compound bound with protein, a compound similar to *S*-alkylmercaptocysteine, according the following formulae.

2. Effect of Inhibitors of Protein-SH Groups

The findings given above suggest that the compound bound with protein is not an additional complex as assumed by Fujiwara but a compound chemically bound with the SH groups of protein, as have been proved by the authors in the cases of TDS and BTDS. For further confirmation, the effects of various inhibitors of the SH-groups were investigated.

Effect of Iodine Oxidation—Urea-denatured protein, 250 mg, was oxidized with 0.1–0.2 ml of 0.1 *N* iodine solution. After the reaction of the oxidized protein with I or II, the amount of the $\text{C}_3\text{H}_7\text{SH}$ bound with protein was determined. As shown in Fig. 3, the amount of the mercaptan bound with protein was decreased according to the increase of iodine.

Inhibition by PCMB—Native or denatured protein was treated with a certain amount of 1.6×10^{-2} *M* *p*-chloromercuribenzoate (PCMB) solution, incubated with I or II and the protein-bound compound thus formed was determined. As shown in Fig 4, the formation of protein-bound mercaptan was markedly decreased with the increase of the blocking of SH-groups. These findings indicate that

FIG. 3 Effect of Oxidation with Iodine
Reaction: 250 mg denatured protein $\rightarrow$ oxidized $\rightarrow$ + 12 mg I or II; pH 7.4; 37° 1 hr.
○, I; ●, II

FIG. 4 Effect of Inhibition by PCMB
Reaction: 250 mg protein $\rightarrow$ block with PCMB $\rightarrow$ + 12 mg I or II; pH 9.0; 37°; 1 hr.
○, I; ●, II; —, denatured; ---, native

the SH- groups of protein are involved in the formation of protein-bound propylmercaptan by the reaction of egg albumin with I or II, and that this complex is of a *S*-propylmercapto-cysteine type.

3. Effects of pH, Time and Temperature

Urea-denatured protein was incubated with I or II in the buffer solution of various pH levels and the amount of propylmercaptan bound with the protein are listed in Table I. As can be seen from the Table, the reaction of the SH-groups of the protein with II in the range of pH 6.0 to 9.0 remains almost the same, but the reaction with I, as in the case with TDS and BTDS, is definitely influenced by the pH level, the bound amount being markedly increased at the pH levels above 7.0. Generally in the SH-disulfide exchange reaction as formula (a), the SH is considered to participate in the reaction with the dissociated form (RS^-). Therefore, in the case of the SH-groups of the protein, its dissociated form (PrS^-) is assumed to be involved in the reaction; the higher the pH, the greater the concentration of PrS^- with the proportional rise in reactivity.

Reaction Time and Temperature — In the formation of the complex like $PrSSC_3H_7$, the reaction is naturally assumed to depend on the temperature and

TABLE I
Effect of pH

Disulfide	pH levels							
	3.0	5.0	6.2	7.5	7.8	8.2	8.6	9.0
	$\mu g\ C_3H_7SH\ bound\ with\ 250mg\ protein$							
I	7	18	52	109	157	253	270	252
II	117	113	165	150	116	178	203	175

Reaction: 25 mg protein + 6 g urea + 12 mg I or II; 1 hr.

FIG. 5 Effect of Time and Temperature
Reaction: 250 mg denatured protein + 12 mg
I or II; pH 7.3

○, I; ●, II; ---, 10°; —, 37°

time. The protein was therefore allowed to react at 10° or 37° and pH 7 and the amount of combined propylmercaptan was determined at each interval. As shown in Fig 5, in the reaction with I, the effects of temperature and time are marked even at 37° and it took considerably a long time for reaching the equilibrium state at 10°. In the case of II, however, these effects were observed only within 10 minutes from the start of the reaction, scarcely any increase in the bound form being detected thereafter, indicating that the compound II was more reactive than I towards the SH-groups of the protein. These findings show that the complex with protein is not a mere additional one but the formation of the compound bound by chemical reaction.

4. Effect of Protein Denaturation

The amount of the propylmercaptan bound with protein was markedly increased in the case of I by denaturation of protein, but in the case of II, the complex was produced regardless of denaturation or nativity of the protein. The finding was further studied in detail. Table II shows that in the case of I, similarly to the cases of TDS and BTDS, the amounts of bound and free propylmercaptan rose with the degree of denaturation of the protein, suggesting the process of the equilibrium reaction (a).

TABLE II
Effect of Protein Denaturation

Disulfide	C ₃ H ₇ SH produced	Urea used for protein denaturation (g/15 ml)				
		0	2	3	4	6
		<i>μg bound and free C₃H₇SH produced from 250 mg protein</i>				
I	Free	40	71	177	177	194
	Bound	31	43	59	71	102
II	Free	15	13	18	23	22
	Bound	154	162	180	182	176

Reaction: 250 mg protein + urea + 12 mg I or II, pH 7.3, 1 hr., 37°

On the other hand, compound II reacted also with the thiol groups of the native protein and the amount of the propylmercaptan bound with the protein rose a little after denaturation. The formation of free propylmercaptan was very little, a finding compatible with the process of the reactions (b) and (c).

SUMMARY

Native or urea-denatured egg albumin was allowed to react with C₃H₇SSC₃H₇ (I) or C₃H₇SSOC₃H₇ (II) under a certain condition. The protein was precipitated by ethanol treatment and the unreacted I or II was completely removed by extracting the precipitate several times with hot ethanol. The protein fraction was reduced by cysteine, producing the free propylmercaptan. It is assumed to have been produced from the compound bound with the protein similarly to S-propylmercapto-cysteine. As the microdetermination of propylmercaptan was possible by utilizing dithizon method, the amount of the bound propylmercaptan under various

contions was investigated and the following results were obtained.

1. The formation of the bound form of I was markedly increased by protein denaturation, producing also the free propylmercaptans. The formation of the bound form of II was somewhat increased after denaturation of the protein, but was observed significantly with the native protein. In this case the formation of free propylmercaptan was scarcely observed.

2. The reaction of I with the protein was markedly dependent on pH levels, rising definitely at pH levels above 7.0. On the other hand, the reaction of II was little affected by pH levels, showing almost a constant activity at pH range above 6.0.

3. The reactivity of I or II was increased with the rise of temperature and time.

4. When the SH-groups of the protein were oxidized with iodine or blocked by *p*-chloromercuribenzoate, the reactivity was remarkably reduced.

These findings suggest that the compound I or II, though the reaction mechanisms are different from each other, reacts with the SH-groups of the protein, forming a protein-bound compound like protein-SS-C₃H₇.

ACKNOWLEDGMENT

The authors wish to express their gratitude to Dr. Masao Fujisawa and Norio Sugimoto of our company for their constant encouragement.

REFERENCES

1. Fujiwara, M., Murakami, F., Fujiwara, K., *Vitamins* **17**, 138, (1959); **20**, 136, (1960).
2. Fujiwara, M., *ibid.* **21**, 461, (1960).
3. Utsumi, I., Harada, K., Kohno, K. and Hirao, H., *J. Vitaminol.* **9**, 41, 50, 89, (1963).
4. Cavallito, C.J., Buck, J.S. and Suter, C.M., *J. Amer. Chem. Soc.* **66**, 1952, (1944).
5. Yurugi, S., *Yakugaku Zasshi* **74**, 511, 514, (1954).
6. Yoshimura, M., *Vitamins* **14**, 627, (1958).
7. Sandell, E.B., *Colorimetric Metal Analysis*, 2nd ed., p. 444, Interscience Publishers, Ltd., (1956).